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Spectroscopic study of human leukocytes

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Abstract

FTIR and auto-fluorescence techniques, based on the monitoring of endogenous molecules, are able to discriminate between normal and leukemic white blood cells. MIR spectra were measured at 4 cm^{-1} resolution on 6 batches of normal lymphocytes and leukemic Daudi cells, centrifuged and dried on a BaF_2 window. The finger-print region (800-1800 cm^{-1}) of each spectrum was selected, detrended by subtraction of a straight tangent base line and normalized at the same area. Significant differences were found in the relative absorbance distribution within the range analyzed between normal and malignant cells.

Epifluorescence microscopy techniques were employed on living leukocytes and Daudi cells. Fluorescence was detected by a digital CCD camera. Spectra were measured by an optical multichannel analyzer coupled to the microscope. Fluorescence was located at cytoplasmic-level, thus probably related to the metabolic processes of the cells. Photophysical properties appear different among the normal leukocyte populations and between normal and leukemic cells as far as spatial distribution and light spectrum and intensity is concerned.

KEY WORDS: Leukocytes, leukemia, autofluorescence, infrared.

1. Introduction

The application of new optical technologies to the field of biology and medicine is in continuous evolution. Fluorescence and Fourier Transform Infra-Red (FTIR) techniques, based on the labelling of biological targets by endogenous molecules, may give complementary metabolic and structural information. We are investigating on their use for the discrimination between normal and leukemic lymphocytes in the effort to improve new diagnostic methods in hematology and possibly new insight into the etiology of leukemia.

1.1. *FTIR Spectroscopy*

In spite of the apparent close similarity of the middle infrared (MIR) spectra of biological matter, at least the so called *finger print region* (~800-1800 cm^{-1}) is known to exhibit a good overall discriminant power between different cell types. This result may be not surprising considering that the biochemical information related to phenetic and genetic expression of a specific organism globally tested by a MIR probe is spread and hidden beneath the shape of the spectrum. Going back to 1957 Greenstreet and Norris [1], many years before the advent of FTIR spectroscopy, measured MIR spectra of heat-killed dried bacteria of different genus and species by means of a refractive double-beam spectrometer and were able to attain a good discrimination using a statistical method based on Spearman rank correlation coefficient.

The advent of modern interferometric FTIR technique in conjunction with very sensitive MCT detectors augmented a great deal the specification of MIR-spectroscopy and has become a very sophisticated yet easy-to-use tool for biomedical research. Recently [2] classification of bacteria strains, performed by clustering methods based on Pearson correlation coefficient, has been attained and a database of reference spectra has been proposed which can be used as an easy and safe method for the rapid identification of clinical isolates. Significant differences were also found between normal and malignant tissues and isolated human cells [3-5]. In a very recent paper [6] Malins et Al. documented progressive structural changes in isolated DNA of the normal female breast leading to a pre-malignant cancer-like phenotype and claimed to have the possibility of predicting breast cancer occurrence at early stages of oncogenesis.

Some work has also been made in the field of haematology on *ex-vivo* normal and leukemic white blood cells and culture models. On comparing MIR spectra of single human normal and leukemic lymphocytes obtained by FTIR microspectroscopy, Benedetti et Al. [7] proposed as a discriminant index the ratio of the integrated areas of the transmittance bands related mainly to DNA content (~1080 cm^{-1}) over that identified as proteic components (~1540 cm^{-1}). More recent papers are dealing with the response of different mammalian leukemic cell lines to experimental stimuli. It was shown that FTIR spectroscopy, performed on cell smears, proved able to distinguish between multidrug resistant and sensitive phenotypes in human K562 and hamster LR73 cell lines [8]. Significant spectral differences were also obtained comparing FTIR spectra of undifferentiated and polynucleated FLG 29.1 cell line, used as a model to study osteoclast ontogenesis [9].

In the present paper we report preliminary results

obtained comparing FTIR spectra of human normal leucocytes with those of Daudi leukemic cell line.

1.2 *Natural Fluorescence Spectroscopy*

A large number of biological components exhibit fluorescing properties so that, when irradiated with suitable wavelengths, they give rise to a natural fluorescence emission (NF) in the ultraviolet-visible spectral range.

Some of the fluorophores involved in autofluorescence emission are related to the biomolecules responsible for the structural arrangement of cells and, principally, proteins due to the presence of the aromatic amino acids tryptophan, tyrosine and phenylalanine (emission in the near ultraviolet). Other fluorophores are biomolecules involved in metabolic functions. For example nicotinic and flavinic coenzymes, have a blue and green fluorescence respectively [10].

On this basis, the NF of biological substrates has been considered an important tool for the investigation of morphologic and functional aspects of cells and tissues without interfering with the native cellular environment. Nevertheless very few studies on haematic cell autofluorescence have been done and very little is known on this subject. In Hematology, very few autofluorescence studies have been reported in the literature. They relate mainly to the eosinophil fluorescence emission [11]. Recently the fluorescence properties of human white blood cells have been studied [12]. Microspectrofluorometric analysis, performed on single cells, was able to distinguish lymphocytes, monocytes, neutrophils and eosinophils according to the intensity and spectral shape of the auto-fluorescence emission in the range from 420 to 650 nm. Differences in the intrinsic metabolism seem to be responsible for the fluorescence differences observed.

In this paper we present a preliminary study on the property of autofluorescence spectroscopy to discriminate also between normal lymphocytes and leukemic cells derived from a human stabilized cell line.

2. Material and Methods

Normal lymphocytes, collected from peripheral blood of healthy donors, and leukemic cells derived from the human stabilized cell line Daudi have been analyzed with FTIR and epifluorescence microscopy spectroscopic techniques. Daudi is a cell line of lymphoid nature deriving from patients suffering from lymphoid neoplasms.

Daudi lines were cultured in RPMI 1640 with 10 % FCS, incubated in 37°C, 5 % CO_2 and 100 % humidity and splitted twice a week. Before use cells were resuspended in saline. Venous peripheral blood was collected in hepatin, applied to a 1.077 density gradient by centrifugating at 400 g for 30 min. Cells at interface were collected, washed and resuspended in saline for fluorescence measurements. FTIR spectroscopy was performed on dried stratification of about 10^6 cells obtained upon centrifugation on 13-mm-diameter × 2-mm-thick BaF_2 window housed in a special support fitting the cytoblock.

2.1. *FTIR measurements*

MIR spectra were measured on 6 different batches of normal lymphocytes and Daudi cells using a Shimadzu FTIR spectrophotometer (mod. 8201PC). A clean BaF_2 disc was used as background reference. Interferograms were collected at 4 cm^{-1} nominal resolution in the range 800-4000 cm^{-1} and averaged over 100 scans using Happ-Genzel apodization function. Absorbance spectra were recorded for off-line calculation.

The *finger-print* region (800 – 1800 cm^{-1}) was selected to test the overall difference in the distribution of absorbance bands. At this stage of study no multivariate analysis was employed. By home-developed algorithm a tangent straight base-line was automatically calculated and subtracted from each spectrum. New relative values of spectral absorbance were substituted to the original ones. All the spectra were then normalized in such a way as to have the same area. At each frequency the difference between mean absorbance of normal and Daudi cells was tested by non-parametric Wilkoxon's rank-sum test.

2.2. *Fluorescence measurement*

Microspectrofluorometric analysis of leukocytes was performed by means of an inverted microscope Diaphot (Nikon, Japan). The microscope was equipped with an oil-immersion CF-UV fluor (Nikon, Japan) objective 100x (N.A. 1.30). Excitation light for both microspectrofluorometric and image analysis was provided by a high pressure 100 W mercury lamp HBO (Osram, Germany), in combination with KG1 (Schott, Germany) heat blocking filter and interference filters to select the excitation wavelengths. Fluorescence emission arising from the cells, collected by the objective and passed through a dichroic mirror (DM 400, Nikon), was analyzed by a multispectral digital CCD camera cooled at –20°C (Compuscope CCD800) with 768x512 pixels and a lumogen coating for enhanced UV sensitivity. The CCD camera was equipped with a filter wheel to select up to 8 different spectral bands. For each sample, the fluorescence image excited at about 365 nm was detected for spectral band centered at 450 nm.

The spectra were measured by an optical multichannel analyzer (OMA III, EG & G Princeton

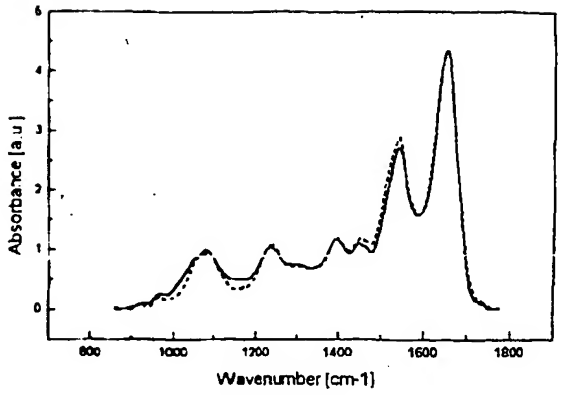

Fig. 1 – FTIR mean absorbance spectra of Daudi cells (dashed line) and lymphocytes (solid line). Significant differences ($p<0.025$) in absorbance are highlighted in gray.

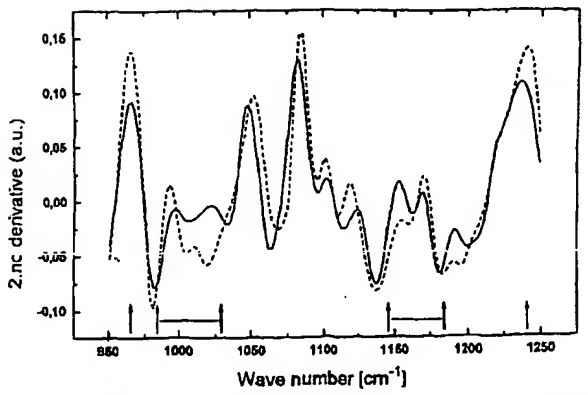

Fig. 2 – Second derivative (multiplied for –1) of absorbance spectra of Daudi cells (dashed) and lymphocytes (solid). The arrows point to the most significant bands.

Applied Research model 1460), connected to an auxiliary output port of the same microscope.

3. Results and Discussion

3.1 *FTIR spectroscopy*

In Fig. 1 are plotted the *finger-print* region mean spectra of Daudi cells (dashed line) and normal lymphocytes (solid line). Significant differences in absorbance were detected by Wilkoxon test mainly between ~900 cm^{-1} and the Amide II region peaked at 1541 cm^{-1}.

In Fig. 2 the means of the second derivative spectra [13] of Daudi cells and lymphocytes in the range [950-1250] cm^{-1} are shown. The most significant ($p<0.005$) differences are found: *i*) in the band peaked at 965 cm^{-1} (assignable to phosphorilated proteins and cellular nucleic acids [14]); *ii*) in the composite region between 980 cm^{-1} and 1030 cm^{-1} (assignable to glycogen and carbohydrates [14]); *iii*) in the composite region between 1140 cm^{-1} and 1180 cm^{-1} (assignable to C-O stretching mode of some amino acids, carbohydrates and membrane lipids [4]); iv) in the band peaked at 1240 cm^{-1} (assignable to nucleic acids backbone asymmetric phosphate stretching but also, in some extent, to amide III).

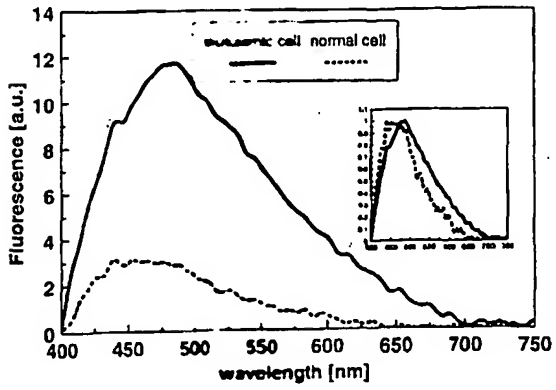

Fig. 3 – Fluorescence emission spectra recorded at microscope on single cell, under excitation at 365 nm and for the same integration time. Can be observed the differences in fluorescence intensity and the shape between normal and leukaemic cell spectra normalized to the maximum (see inset).

3.2 *Fluorescence spectroscopy*

Microfluorometric analysis was performed on single living cell. Typical emission spectra recorded on excitation at 366 are shown in Fig. 3. On excitation at 366 nm, the NF consisted of a large emission band centered in the range 440-460 nm. Additional fluorescence contributions were observed, as shoulders, at longer wavelengths.

Typical examples of the fluorescence patterns exhibited by lymphocyte and leukemic cell on excitation at 366 nm are shown in Fig. 4.

The cell populations examined exhibit peculiar fluorescence emission bands both in the UV and blue-green spectral regions. The blue component (excitation peak ~350 nm, emission peak ~450 nm) can be ascribed to nicotinic coenzymes and to their derivatives, in particular to the reduced form of nicotinamide adenine dinucleotide (NADH) [15,16]. The green component (excitation peak ~430 nm, emission peak ~530 nm) is attributed to flavins and flavin coenzymes, but lipopigments can also contribute [17]. The intracellular fluorescence pattern reveals that fluorescence is located at cytoplasmic-level, the nucleus is almost dark, thus probably related to the metabolic processes of the cells. DAUDI cells exhibited more homogeneous distributed fluorescence. The photophysical properties of cells appear different among the normal populations and between normal and leukaemic ones.

Both the nature and the extent of the differences change according to the excitation wavelength. The intrinsic metabolic engagement, rather than the cell dimension, seems to be responsible for the

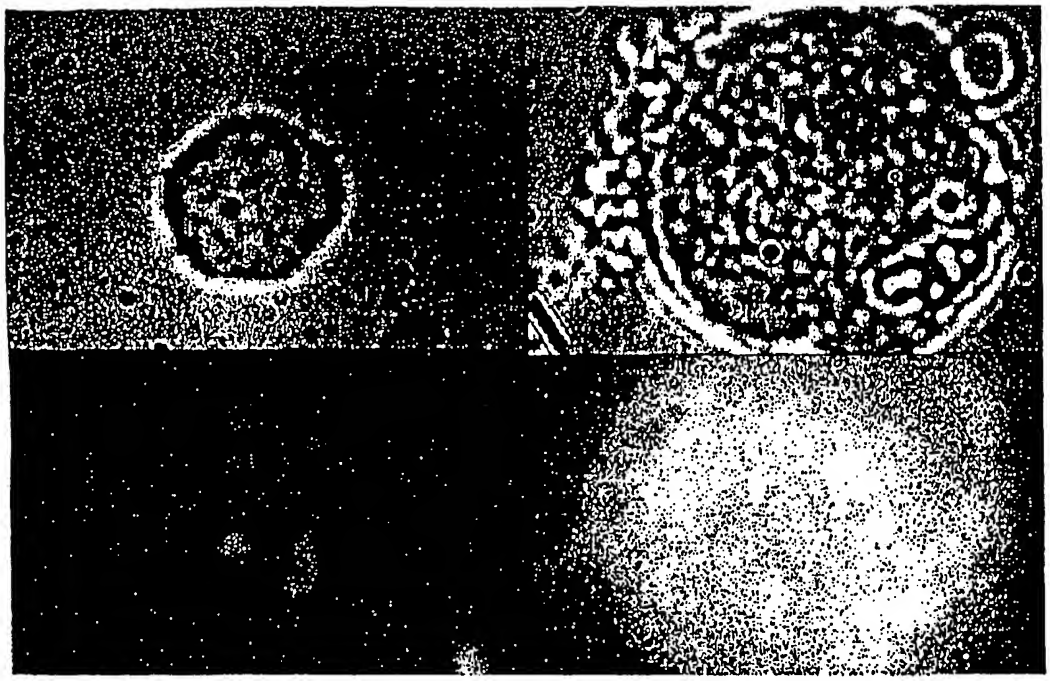

Fig. 4 – Transmission (top) and autofluorescence (bottom) images of lymphocyte (left), and leukaemic cell (right), recorded under excitation at 365nm.

differences observed among the lymphocyte population emissions.

Furthermore, NF of leukemic cells shows peculiar characteristics concerning their responce to UV irradiation. Under excitation, the fluorescence initially arises from the peripheral region of the cell, and it appears as a blue-green ring very close to the membrane. Progressively the thickness of the ring increases until it occupies all the cell. [see Fig. 5]. At the same time the intensity also increases. The leukemic cell spectrum, in comparison with leukocyte one, reveals some differences: the peak in the blue band is red shifted and a broadness of the green band is evident [Fig. 3 inset].

4. Conclusion

In this preliminary study, the number of samples analyzed and compared by FTIR and fluorescence spectroscopy is small.

Neverthless FTIR results indicate that it is even possible to find or to assume structural differences in the comparison of two groups of cells and assign them tentatively to important cellular components such as carbohydrates, phospholipids and nucleic acids. We did not calculate any discriminant function but we are very confident to confirm also in our case the overall discrimination power of FTIR spectroscopy, not only between normal and malignant cells, but also for normal blood cells classification.

On the other side fluorescence results clearly show the existence of a link between photophysical properties and cell metabolism. Changes in the state of cells and tissues, occurring during physiological processes or in connection with pathological conditions, are supposed to modify both the amount and distribution of the natural fluorophores. On this basis, the NF study may provide new insight into metabolic processes and biochemical pathways.

Considering the statistical significance of the data collected and the relatively simple method of measurement, interesting prospects have been opened for applying both FTIR and autofluorescence techniques to the detection of abnormal and immature hemopoietic cell.

In conclusion we think that combining MIR and NF techniques may give complementary information on structural and functional properties of blood cells so that not only automatic discrimination can be possible but also new insight into cancer development dynamics and early diagnosis could be reached.

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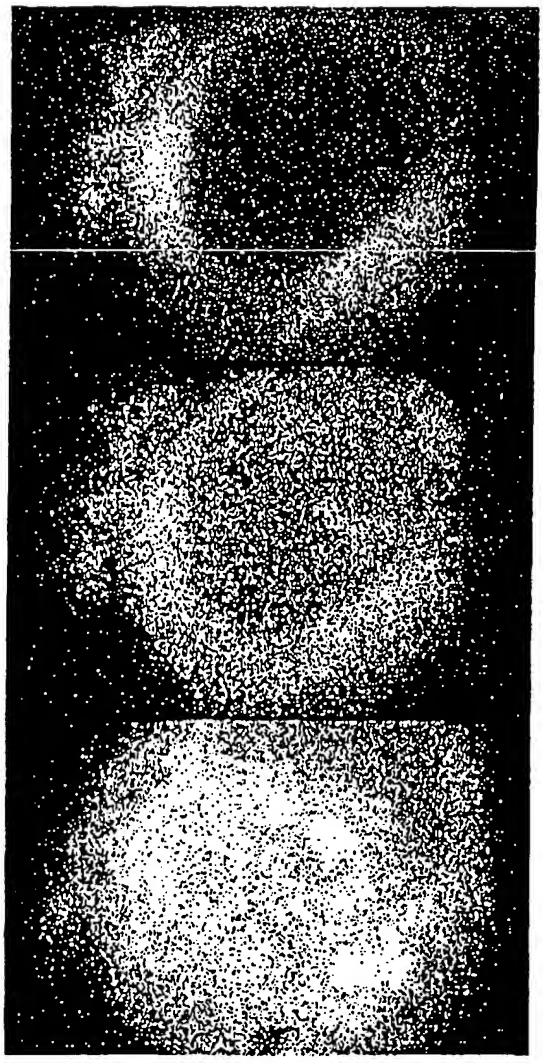

Fig. 5 – Autofluorescence images of a single DAUDI cell excited at 365nm and recorded after increasing time (from top to bottom) of UV irradiation. Upper image was recorded after 2 sec of exposure at 365nm. Middle and bottom images were recorded after 20 sec and 60 sec, respectively.

REFERENCES

[1] Greenstreet JES and Norris KP. The existence of differences between the infra-red absorption spectra of bacteria. Spectrochim Acta 1957: 9; 177-98.

[2] Naumann D, Helm D and Labischinski H. Microbiological characterizations by FT-IR spectroscopy. Nature 1991: 351; 81-2.

[3] Rigas B and Wong PTT. Human colon adenocarcinoma cell lines display infrared spectroscopic features of malignant colon tissues. Cancer Res 1992: 52; 84-8.

[4] Wong PTT, Goldstein SM, Grekin RC, Godwin TA, Pivik C and Rigas B. Distinct infrared spectroscopic patterns of human basal cell carcinoma of the skin. Cancer Res 1993: 53; 762-5.

[5] Romano S, Balzi M, Dei L, Lerman A, Neuberger W and Becciolini A. Differences in sugar phosphate bands between normal bladder mucosa and tumoral tissue detected by FTIR microreflectance spectroscopy. In: Optical and Imaging Techniques in Biomedicine. Lewis A, Robert-Nicoud M, Schneckenburger H, Wilson A. Ed. SPIE 1995: 2329; 201-7.

[6] Malins DC, Polissar NL, Nishikida K, Holmes EH, Gardner HS and Gunselman SJ. The etiology and prediction of breast cancer. Fourier transform-infrared spectroscopy reveals progressive alterations in breast DNA leading to a cancer-like phenotype in a high proportion of normal women. Cancer 1995: 75; 503-17.

[7] Benedetti E, Vergamini P and Spremolla G. FT-IR analysis of single human normal and leukemic lymphocytes. Mikrochim Acta [Wien] 1988: I; 139-41.

[8] Le Gal JM, Morjani H and Manfait M. Ultrastructural appraisal of the multidrug resistance in K562 and LR73 cell lines from Fourier transform infrared spectroscopy. Cancer Res 1993: 53; 3681-6.

[9] Romano S, Benvenuti S, Conti A, Benedetti En, Bramanti E, Rossi I, Benedetti Ed. Fourier transform infrared microspectroscopy shows significant differences between spectra of undifferentiated and polynucleated FLG29.1 dried cells. In: Microscopy, Holography, and Interferometry in Biomedicine. Fercher AF, Lewis A, Podbielska H, Schneckenburger H, Wilson T Ed. SPIE 1994: 2083; 195-200.

[10] Chance B. Microspectroscopy and flow cytometry. In: Cell Structure and Function by Microspectrofluorometry. Kohen E and Hirschberg JG Ed. San Diego. Academic Press 1989; 53-69.

[11] Barnes D, Aggarwal S, Thomsen S, Fitzmaurice M and Richards-Kortum R. A characterization of the fluorescent properties of circulating human eosinophils. Photochem Photobiol 1993: 58; 297-303.

[12] Monici M, Pratesi R, Bernabei PA, Caporale R, Rossi Ferrini P, Croce AC, Balzarini P and Bottiroli G. Natural fluorescence of white blood cells: spectroscopic and imaging study. J Photochem Photobiol (B) 1995: 30; 29-37.

[13] Susi H and Byler M. Protein structure by Fourier transform infrared spectroscopy: second derivative spectra. Biochem Biophys Res Comm 1983: 115; 391-7.

[14] Wong PTT, Wong RK, Caputo TA, Godwin TA and Rigas B. Infrared spectroscopy of exfoliated human cervical cells: Evidence of extensive structural changes during carcinogenesis. Proc Natl Acad Sci USA 1991: 88; 10988-92.

[15] Galeotti T, Van Rossum GDV, Mayer DH and Chance B. On the fluorescence of NAD(P)H in whole cell preparations of tumors and normal tissues, Eur J Biochem 1970: 17; 485-96.

[16] Salmon JM, Kohen E, Viallet P, Hirschberg JG, Wouters AW, Kohen C and Torell B. Microspectrofluorometric approach to the study of free/bound NAD(P)H ratio as metabolic indicator in various cell types. Photochem Photobiol 1982: 36; 585-93.

[17] Benson RC, Meyer RA, Zaruba ME and McKhann GM. Cellular autofluorescence – Is it due to flavins?. J Istochem Cytochem 1979: 27; 44-8.